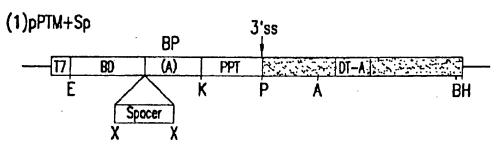
Delivered Therapeutic Gene Splice Site Spacer Binding Domain 5.

FIG. 1



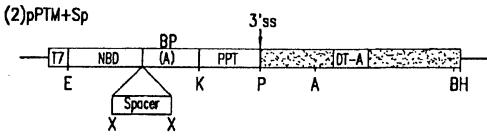


FIG.1B

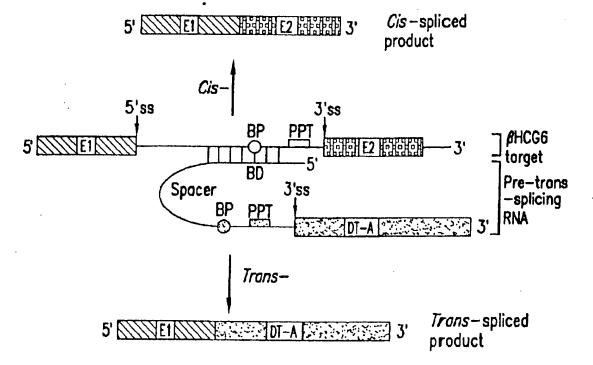


FIG.1C

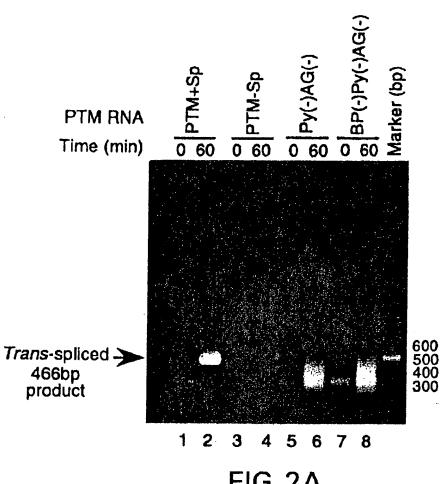
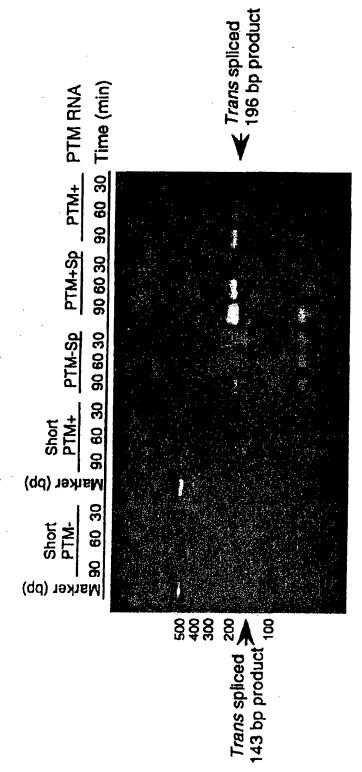


FIG.2A



1716151413121110 9 8 7 6 5 4 3 2 1

FIG.2B

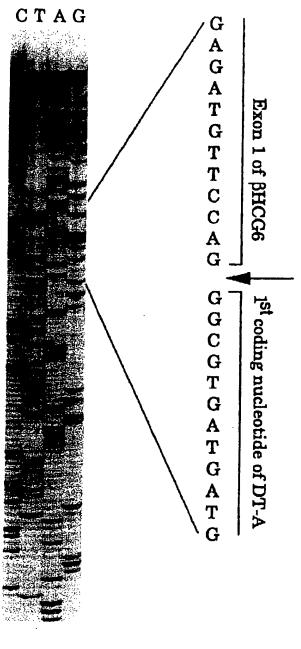


FIG.3

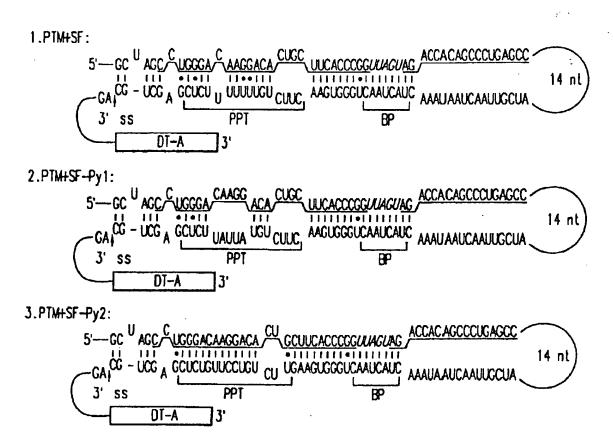


FIG.4A

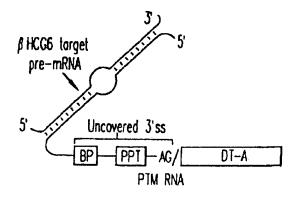


FIG.4B

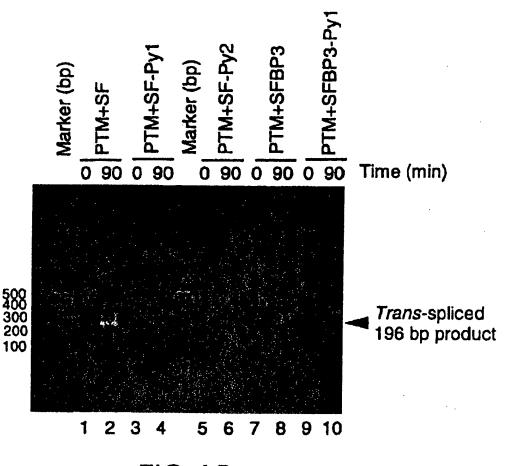
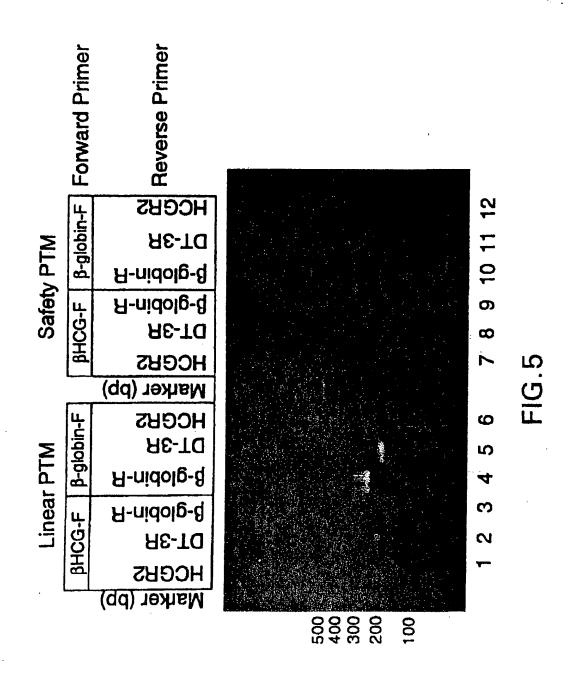
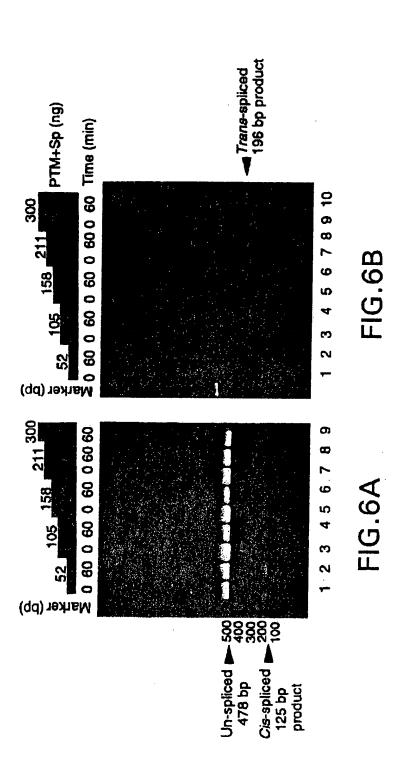
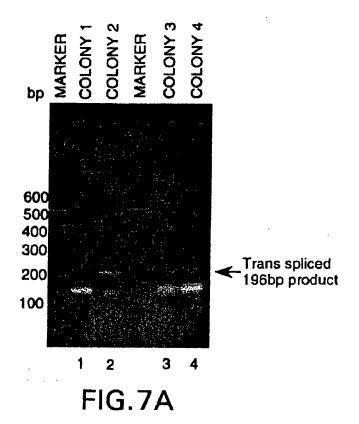


FIG.4C



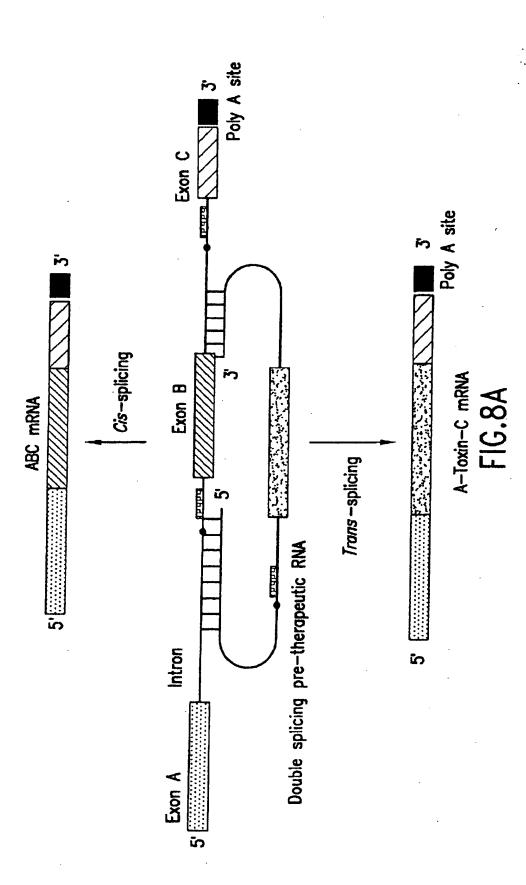


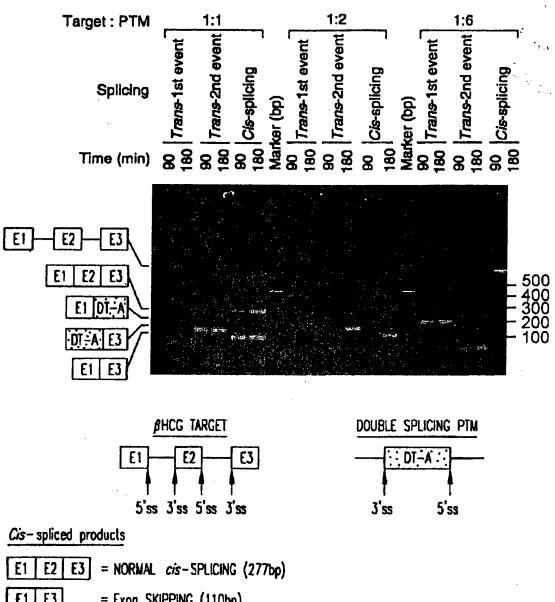
des



1ST CODING NUCLEOTIDE OF DT-A GATTCTTCTTAAATCTTTTGTGATGGAAAACTTTTCTTCGTACCACGGGACTA EXON 1 OF PHOGE | 5-CAGGGGAGGACGATGATGATGTTCCAG-GGGCTGATGATGTTGTT AACCTGGTTATGTAGATTCCATTCAAAA-3'

FIG.7E





## *Trans*- spliced products

= 1st EVENT, 196bp. Trans-SPLICING BETWEEN 5' ss OF TARGET & 3' ss OF PTM.

= 2nd EVENT, 161bp. Trans- SPLICING BETWEEN 3' ss OF TARGET & 5' ss OF PTM.

FIG.8B

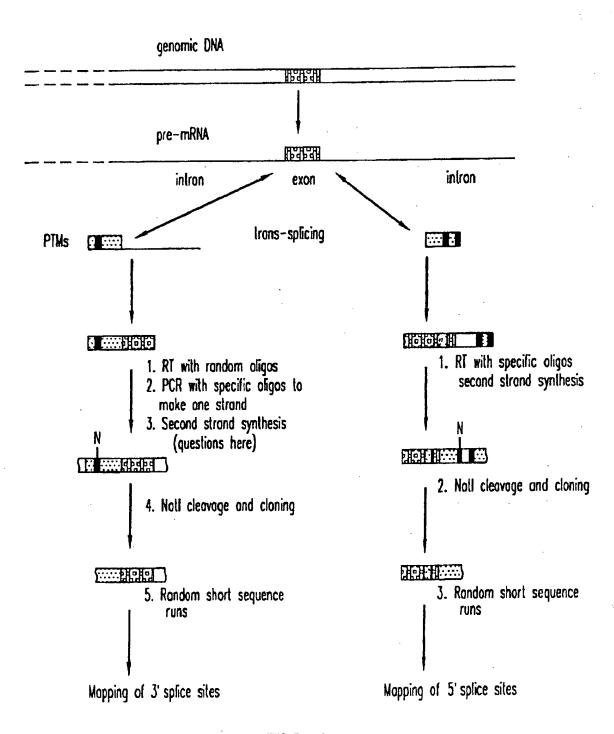
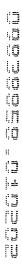
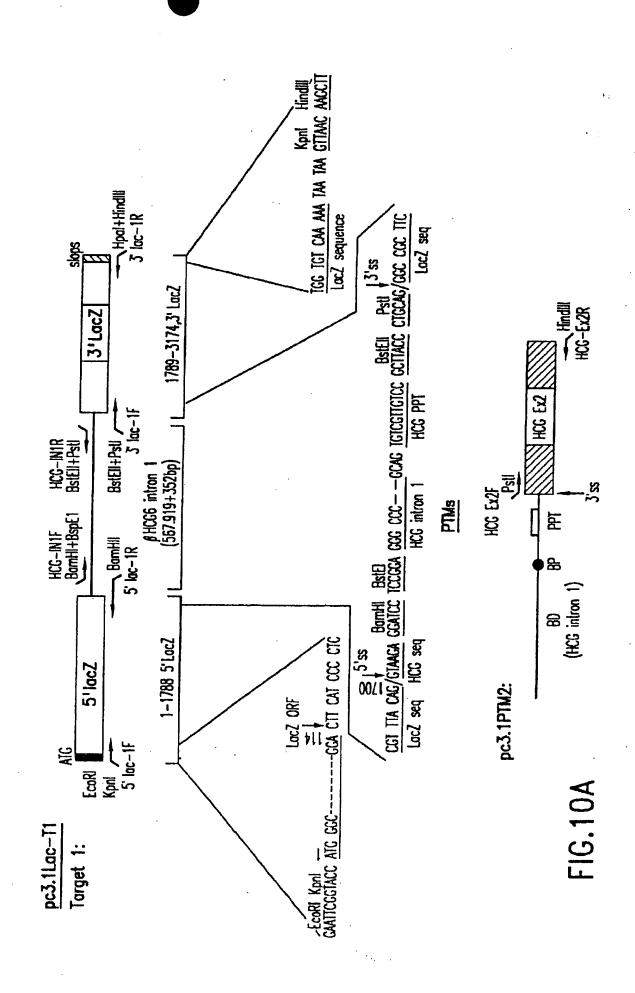


FIG.9





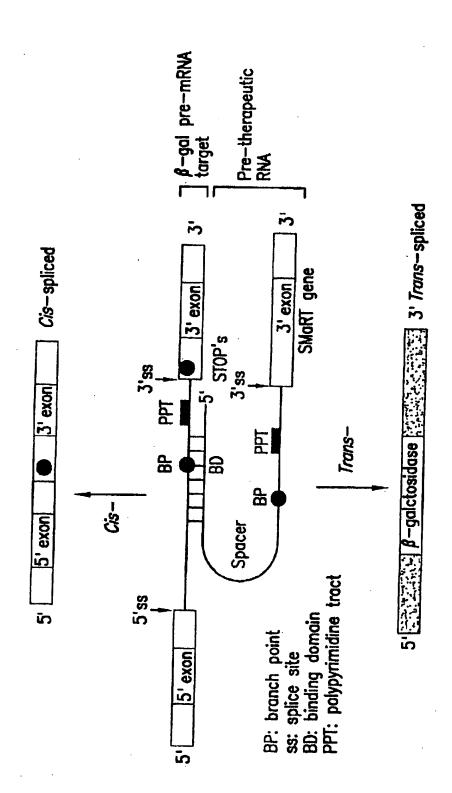


FIG. 10B

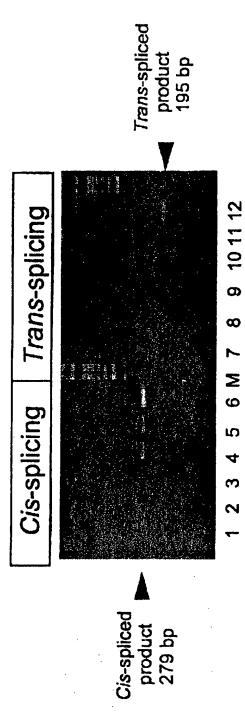


FIG.11A

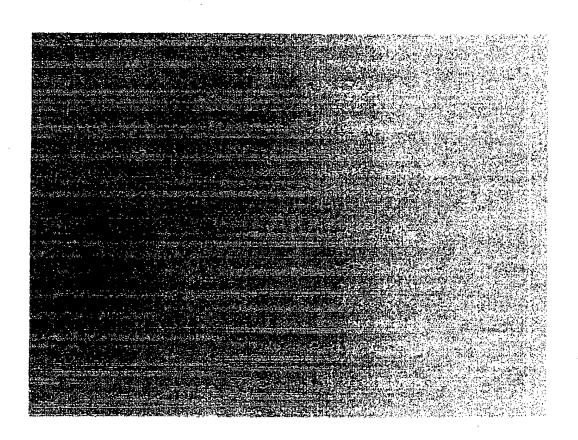


FIG.11B

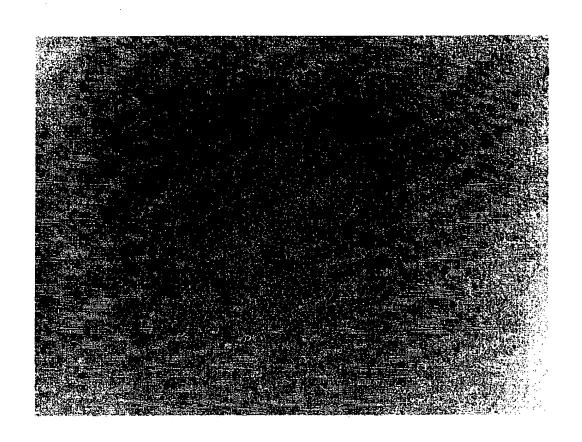


FIG.11C

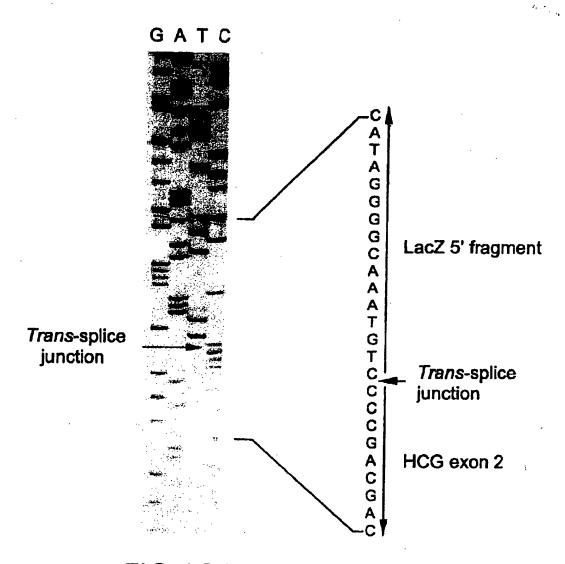


FIG.12A

1. NUCLEOTIDE SEQUENCES OF THE cis-SPLICED PRODUCT (285 bp): BioLac—TR1

GOCTTTCCCTACCTCCACACACCCCCCCTCTTCCCATTCCCAATACCCCCACCCACCCATGCGTAACACTCTTC

GOOGTITCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCCCCGTTTACAG/GGCGGCCTTCGTCTAATAATG Splice junction

GGACTGGGTGGATCAGTCGCTGATTAAATATGATGAAAACGCCAACCCGTGGTCGGCTTACGCCGTGATT1

TGGCGATACCCCCAACCAACCACTTCTGTATGAACCGTCTGGTCTTTGCCCACCCCACCCCCAC Lac-TR2

2. NUCLEOTIDE SEQUENCES OF THE trans-SPLICED PRODUCT (195 bp)

BioLac-TR1

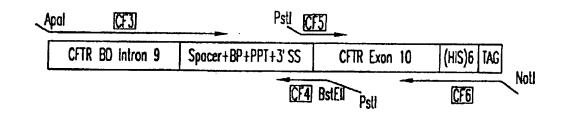
GCCTTTCCCTACCTGGAGAGACCCCCCCTGATCCTTTGCCAATACCCCACGCCATGCCTAACACTCTTGC

COGITICOCTAMAIACICOCAGGCGITICOICAGIAICCCCCTITACAG/COGCTGCIGCIGCTGCTGCTGCT Splice junction

GAGCATGGGGGGACATGGGCATCCAAGGAGCCACTTGGGCCAGGGCGAGGTGCGG

FIG. 12B

## CFTR Pre-therapeutic molecule (PTM or "bullet")



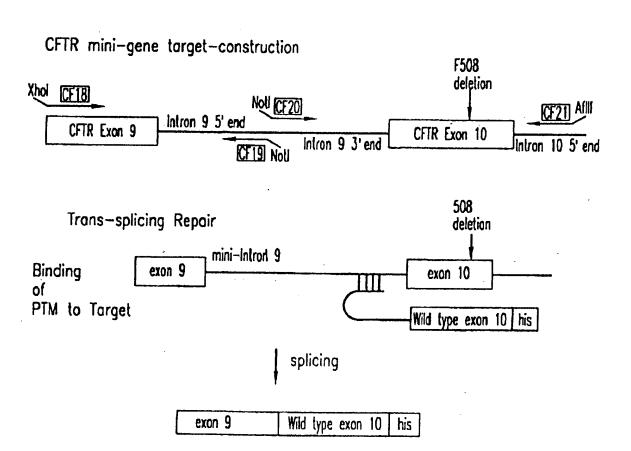
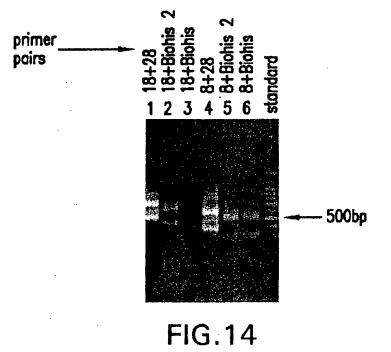


FIG.13



240 GCTAGCG TTAAACGGCCCACCATCATTAGGTCATTATCCCCGGAACATTATTATAACGTTGCTCGAGTACTAAC 80 STOP F508 TICCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTG ATTOCTGTCACCTTCTTAAAGTAAGACAAGAGTCAAAAGGACCTAATAOGGACCGTGGTAATTTCTTTTATAGTAGAAAC ACCATGGAGAAGAAAAAAAGGACGTCHGAAGTGAAGATTACTACTAATACCCTCTTGACCTCGGAAGTCTCCCATTTTA CGATCGCAAATTTGCCCCGGTGGGTAGTAATAATCCAGTAATAGCCGCCTTGTAATAATATTGCAACGAGCTCATGATTG Sca I Positions of Restriction Endonucleases sites (unique sites underlined) GCTAGCGTTTAA ... TGCCACTCCCAC linear BINDING DOMAIN Sph TAAGCACAGTGGAAGAATTTCATTCTGTTGTCAGTT INTRON 9 BD Dde 1 190 102 500 b.p. Hae III Sau96 Sau96 I Ban II Apa I Xmn **FCCTCTTCT DNA** sequence Nhe I Dra I

FIG. 15A

320

GIGITICCIATGATGATATAGATACAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGCATCATCATCATCATTAG

CACAAAGGATACTACTTATGTGTGTTTCCAGTAGTTTCGTACGTTGATCTTCTCGTAGTAGTAGTAGTAGTAATC

\$\frac{\sqrt{1}}{\text{Ban II}}\$\$Sau3A I \\ \text{Dpn I} \\ \text{BamH I} \\ \text{Kpn I} \\ \text{ACTAGTGCATCCCAGCTTCGAACTT A00} \\ \text{IGATCACCTAGGCTCCGCTACCTTCGAATTCAA} \\ \text{CF2R} \\ \text{II} \\ \text{Spg} \\ \text{372} \\ \text{373} \\ \text{373} \\ \text{373} \\ \text{390}	378 PRESENT IN PTM 3' UT 378 BUT NOT TARGET
Sac 1   Sau 11   Pst 1   Dpn 1   HinD 111   Droper	Sau3A I
Hae 111 Not 1                                   323	S C

CF27

410

CTCCAACCTCCCAC 500 CACCTTCCACCTCACCTC

						<b>!</b>		
	7	۰	- 1	_	٠ ١	1	1	
	Sau96	Sca 1	Sma 1	Soh 1	S	Ssp I	Stu I	
ases site usage	1	-		ı	7	1	J	
	Nde I	Nhe I	Not I	Pf IM I	Pst 1	Pvu ]	- Pvu 11	
donucle	_		t	7	I	-	i	
Striction Er	<ul><li>EcoR I</li></ul>	EcoR V	Hae II	Hoe III	HinC 11	Hind III	Hinf I	
9 2 2 3	ı		ı	J	-	7	1	
•	Acc I	Apa I	Apal I	Avr II	BamH 1	Bon II	Bbe I	

15B

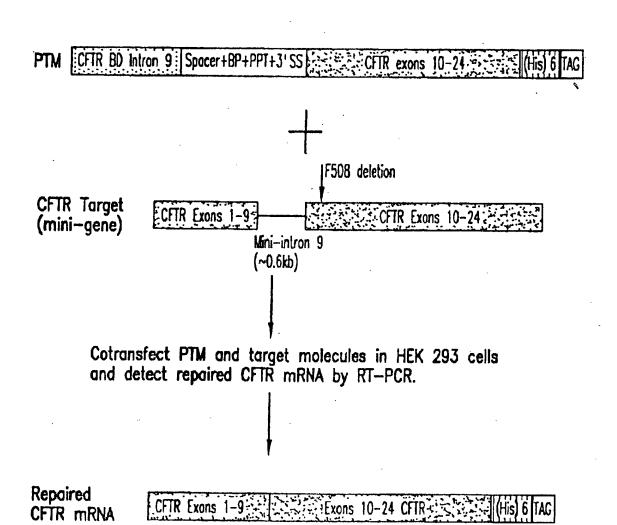


FIG.16

Double Splicing PTM

CFTR BD intron 9 spacer+BP+PPT+3'SS CFTR exon 10 Spacer+BP+PPT+5'SS CFTR BD intron 10

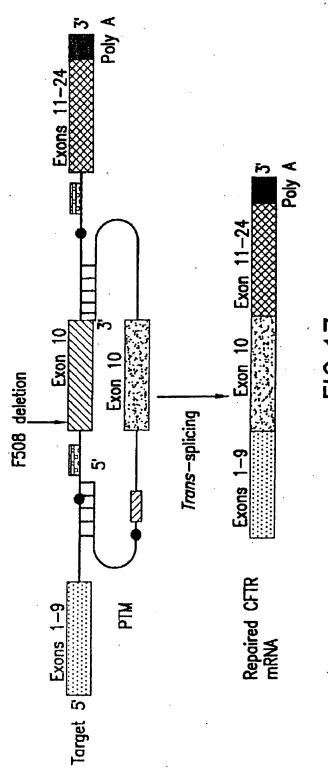


FIG. 17

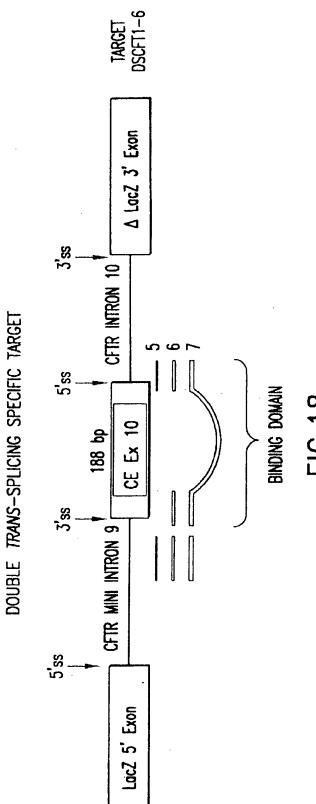


FIG. 18

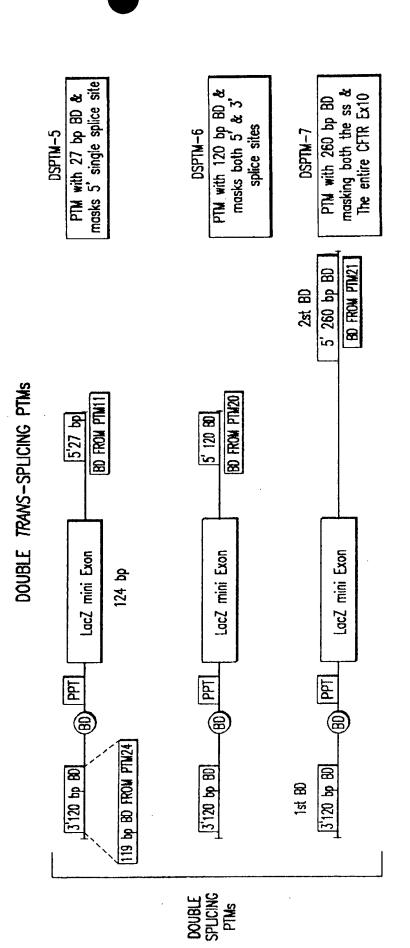
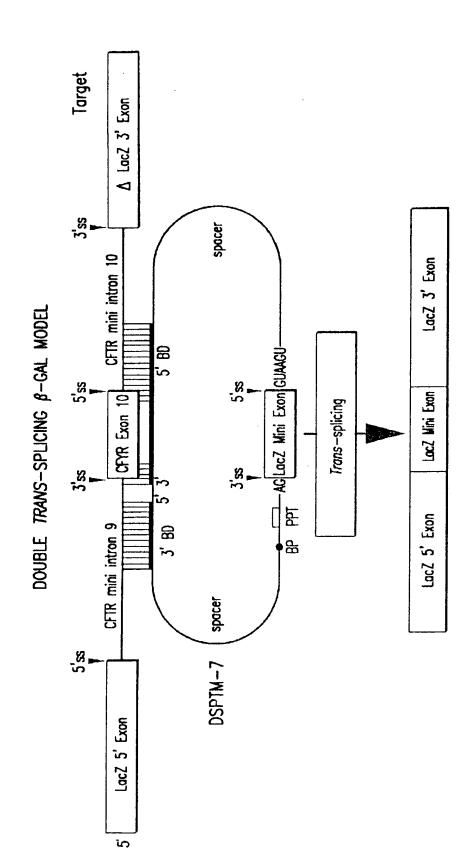
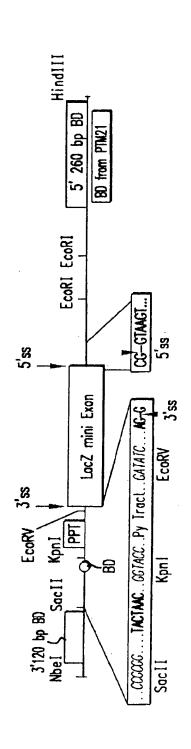


FIG. 1



Repaired LacZ mRNA



(1) 3' BD (120 BP): GATICACTIGCICCAAITAICAICCTAAGCAGAAGTGTATATICTIATTIGTAAAGATICTATTAACTCATTIGATTC AAAATATTTAAAATACTTCCTGTTTCATACTCTGCTATGCAC

(2) Spacer sequences (24 bp): AACAIIATIATAACCTIGCTCCAA

3'ss LacZ mini ★ exon (3) Branch point, pyrimidine tract and acceptor splice site: TACIAAC I GGTACC ICTICITITITITI GAIAIC CIGCAG &CC GGC

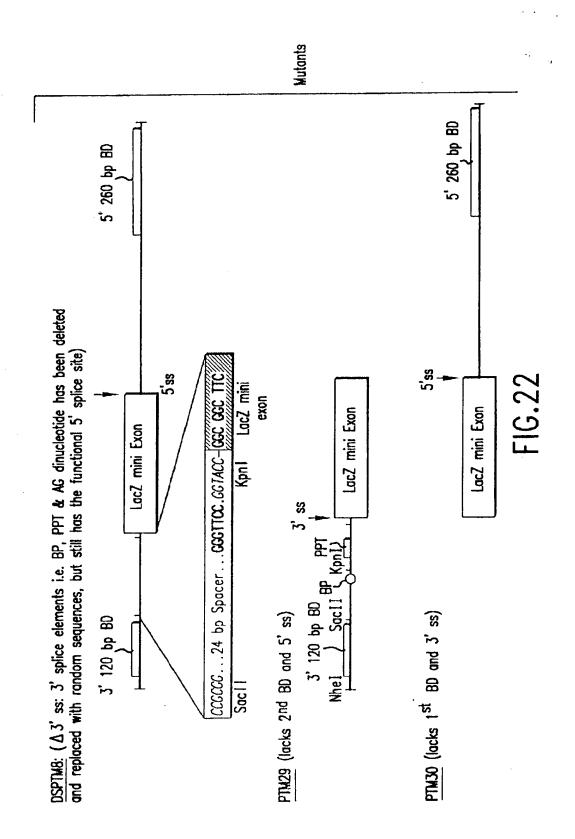
exon Poly Carrier commence of the ACC CARACT CITATOR CARACT

(4) 5' donor site and 2<sup>nd</sup> spacer sequence:| TGA ACG|CIAACI GITAICACCCAIAIGICICIAACCIGAIICGGGCCIICGAIACG CTAAGATCCACCGG

BD (260 BP): ICAAAAAGIITICACAIAAITICIIACCICIICIICAAAIICAIGCIIIGAIGACGCIICIGIAICIAIAIICAICAIIGGAA ACACCAATGATTTTCTTTAATGGTGCCTGGCATAATCCTGGAAAACTGATAACAATGAAATTCTTCCACTGTGCTTAA AAAAACCCTCTGAATTCTCCCATTTCTCCCATAATCATCATTACAACTGAACTCTGGAATAAAAACCCATCATTATTAACTCA

(2) 2,

FIG.21



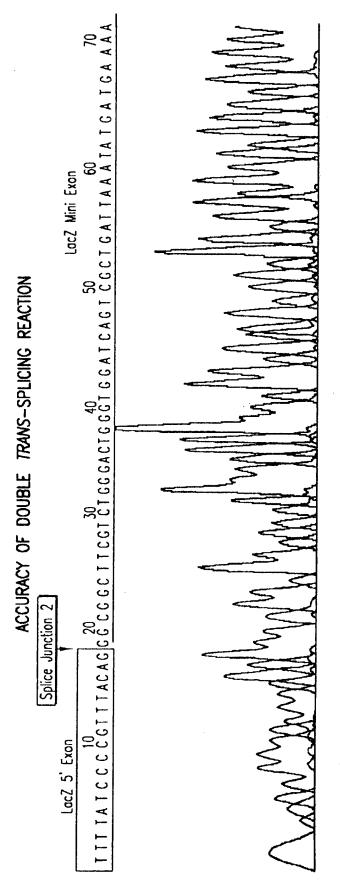


FIG.23A



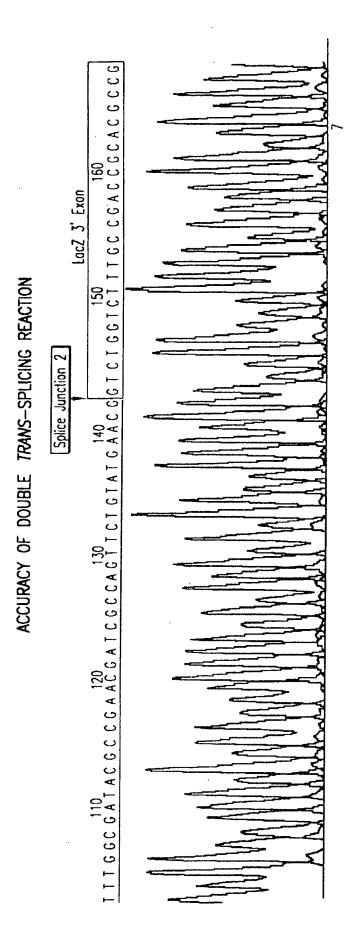


FIG.23B

(120 kDa)

Lane 1: DSCFT1.6 Target alone Lane 2: DSPTM7 Lane 5: Delta 3' splice mutant alone \_ane 3 Target + PTM #6 \_ane 4: Target + PTM #9

25 µg 25 µg 25 µg 25 µg 25 µg 25 µg 25 µg

Lane 7: Target+PTM29+30 (mutants)

\_ane 6: Target + Delta 3' ss

Figure 24

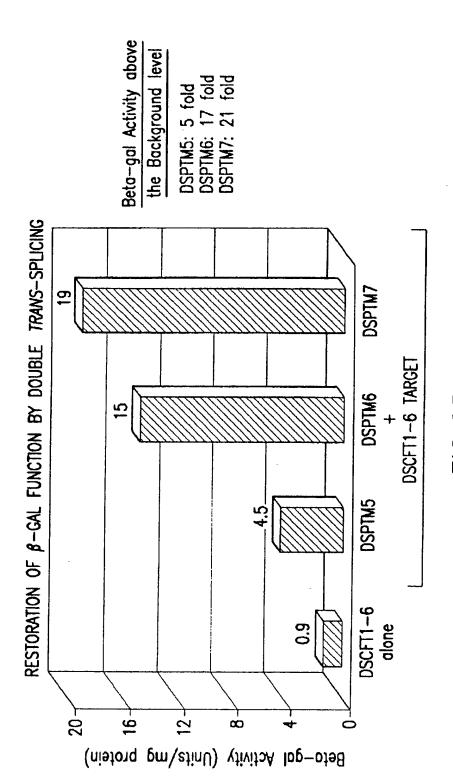


FIG.25

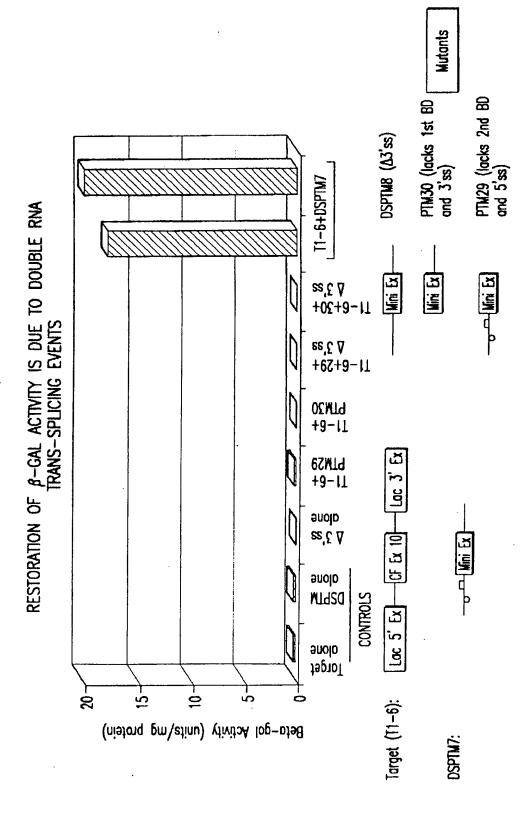


FIG. 26

CED212CFD

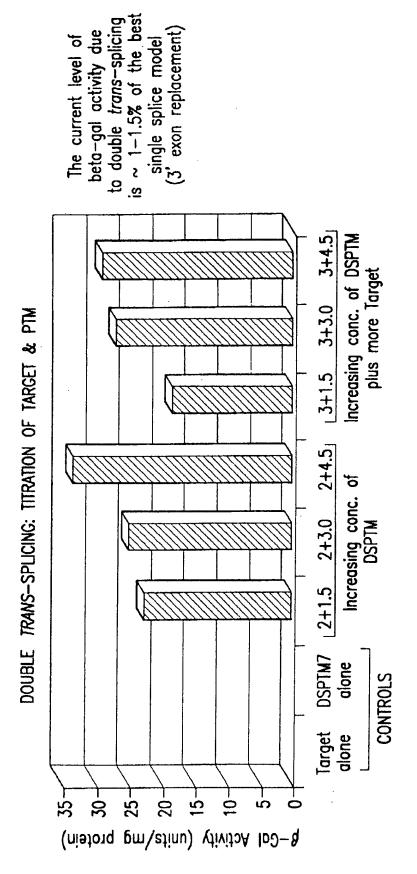
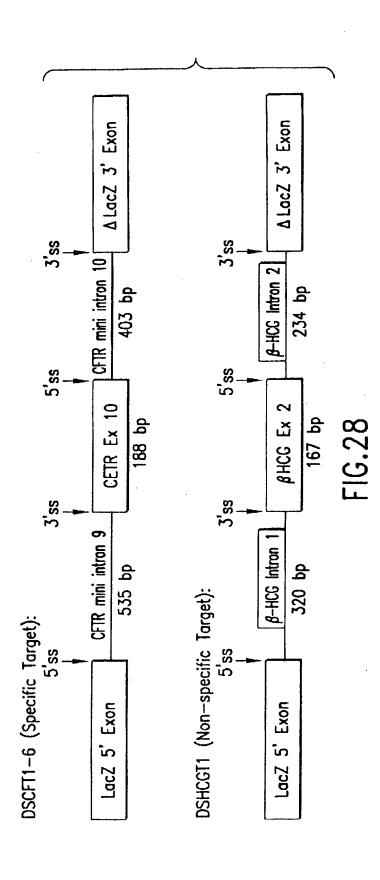


FIG.27



### SPECIFICITY OF DOUBLE TRANS-SPLICING REACTION

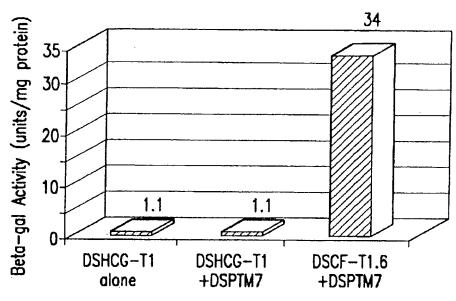


FIG.29



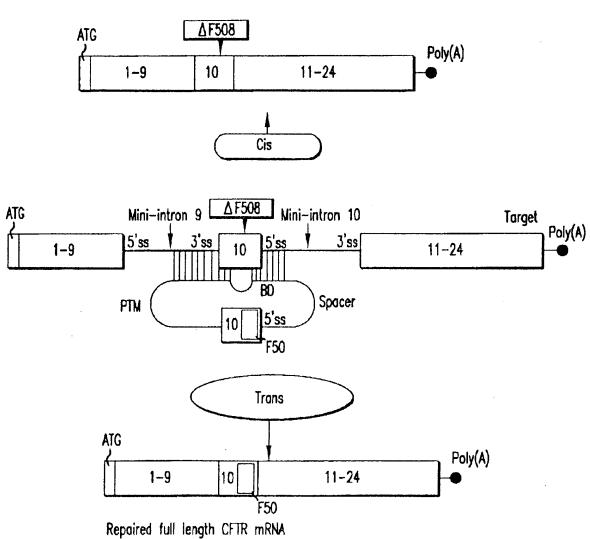
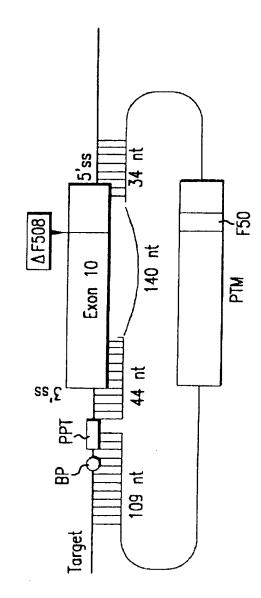


FIG.30

PTM with a long binding domain masking two splice sites and part of exon 10 in a mini-gene target



<u>Ç 7 TCCCCCTCAGTTACCACCAGTACCCCTATCCCTCC</u>CTGAT<u>TAAG</u>CCCTC<u>TCAGTTG</u>CAGGAC A<mark>CCAGCT</mark>IGCTÇATGATGATGATGGGGTTAGA<u>ACCAAGT</u>GA<u>A</u>GGGQAAGATÇAAACA<u>TTCC</u>G G<u>CCGCATCAGC</u>TI<u>I</u>TGCAGCCAATTCAGTI</u>GGATCATGCCCCGGTACCATCAAGGAGAACATAAT MCU in exon 10 of PTM 88 OF 192 (46%) bases in PTM exon 10 are not complementary to its binding domain (bold and underlined).

FIG.31

## Sequence of a double *Trans*—spliced product

iiii

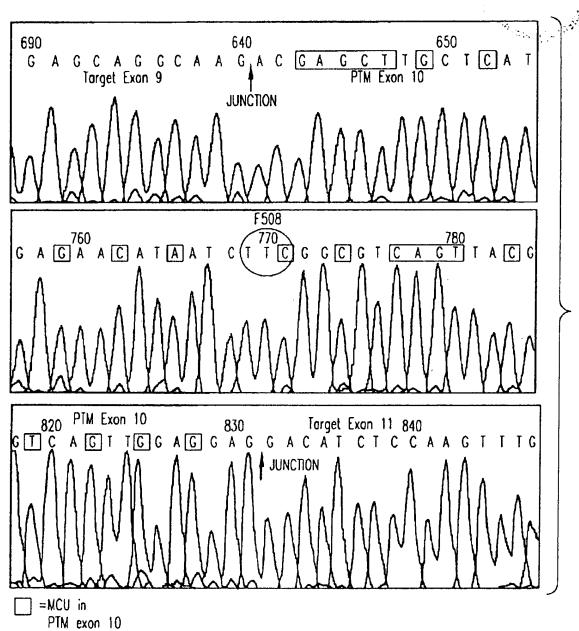
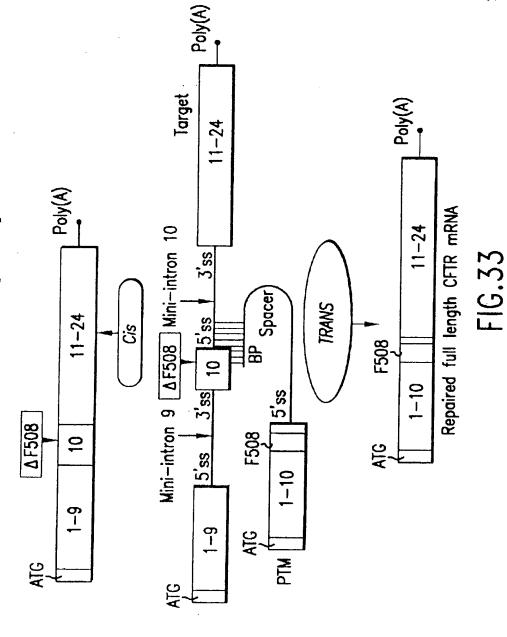


FIG.32

CF—TR Repair: 5' Exon—Replacement schematic diagram of a PTM binding to the splices site of intron 10 of a mini—gene target



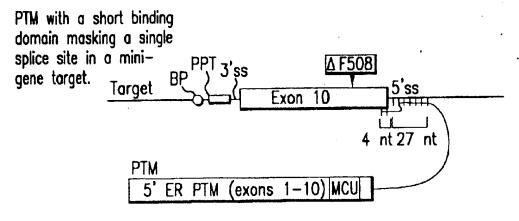


FIG.34A

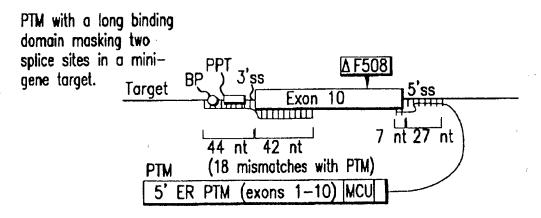


FIG.34B

PTM with a long binding domain masking two splice sites and the whole of exon 10 in a mini—gene target.

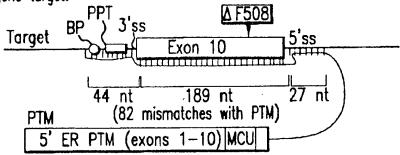
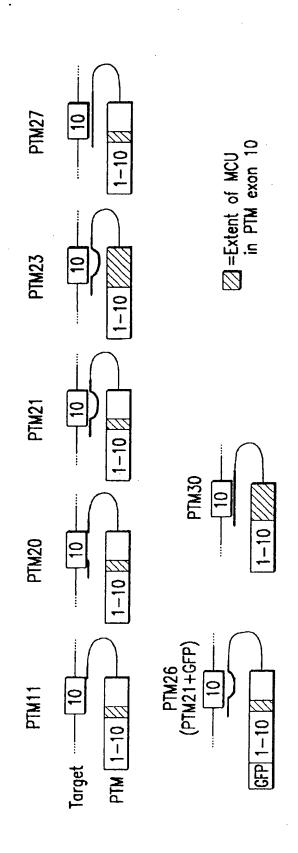


FIG.34C



MCU in exon 10 of PTM 88 of 192 (46%) bases in PTM exon 10 are not complemetary to its binding domain.

ACCACCTTCCTCATCATCATCCTCCACCCACTTACAACTCAAGTCAAGCCAAGATCAAACATTCCC GCCGCATCAGCTTTTGCAGCCAATTCAGTTGGATCATGCCCCGGTACCATCAAGGAGAAGATAAT <u>Ç 77CGGCGTCAGTI</u>AÇGAÇGAĞTA<u>CCG</u>ÇTA<u>ICGCTCG</u>GTGATIAAGGCÇTG<u>ICAG</u>T

# FIG.35

Target

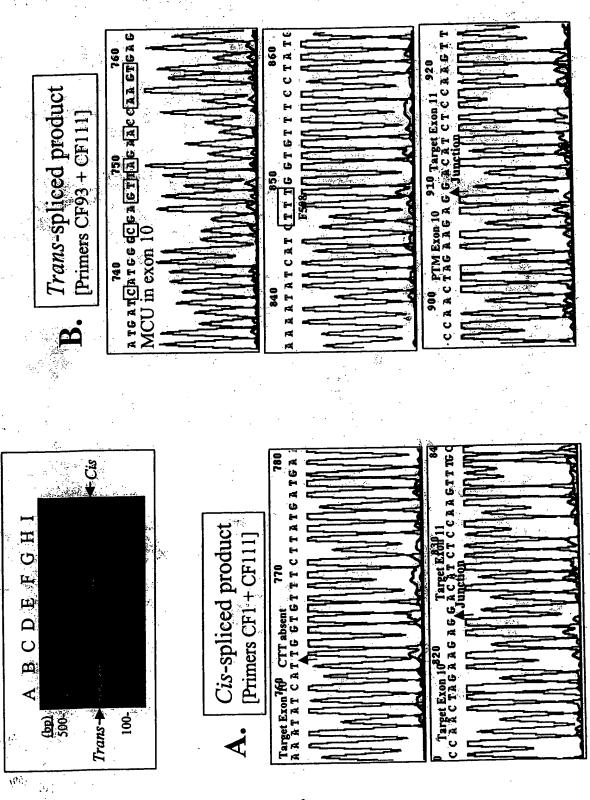


Figure 36

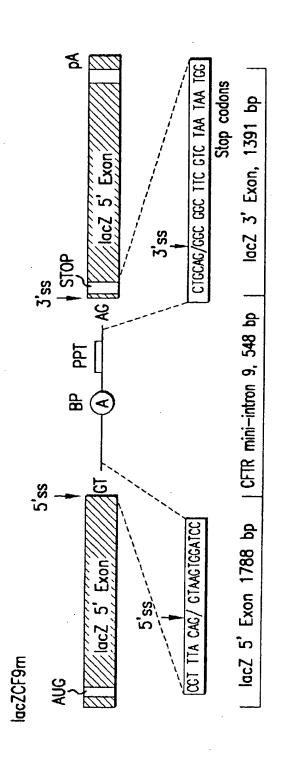


FIG.37A



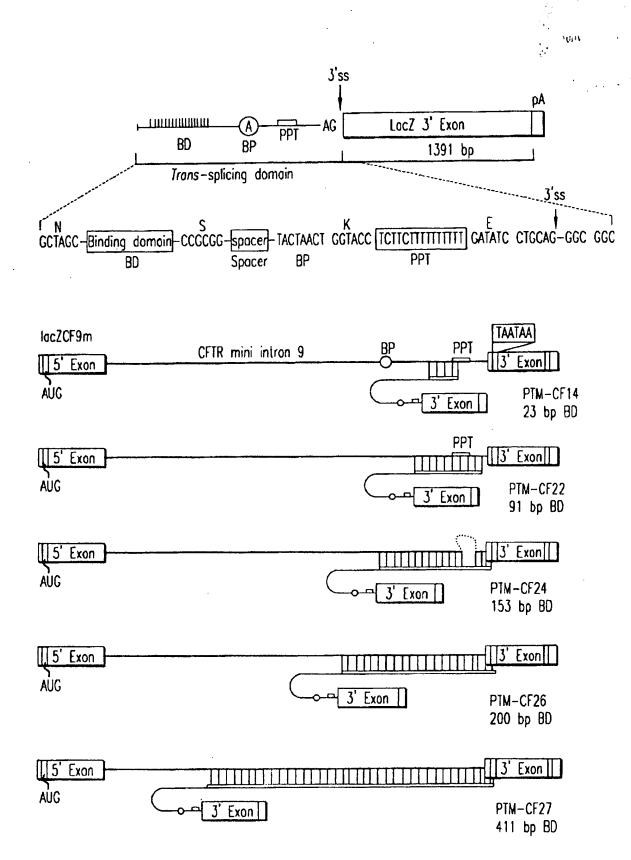
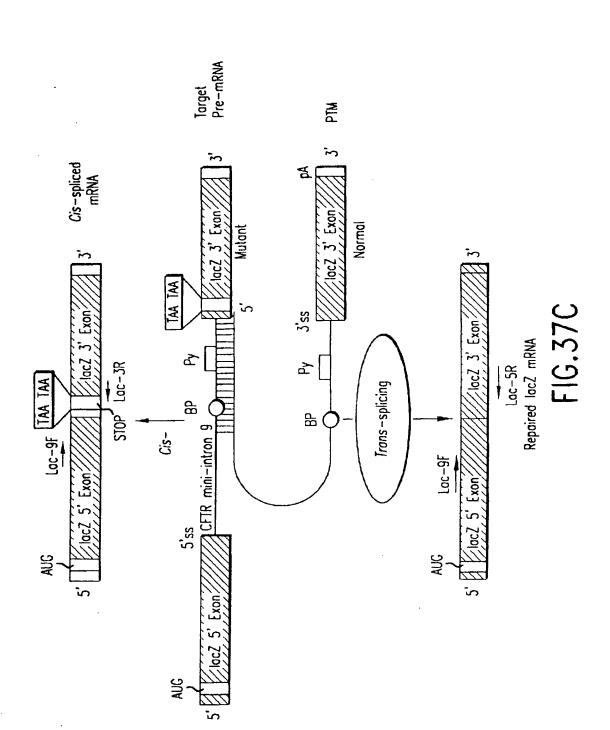


FIG.37B



Trans-spliced (299 bp) # PCR cycles Total RNA # PCR cycles Total RNA 副订章 2 15 20 25 30 50 ng lacZCF9 20 25 25 ag lac7.CF9 Trans-splicing 9 10 11 12 M 1212 Trans-splicing 30 100 ng 20 25 30 200 mg 20 25 38 A lacZCF9n1 + PTM-CF24 30 Figure lacZCF9r. + PTM-CF14 20 25 30 50 ng 20 25 100 ng \_ ک ہ 25 30 Si) iig 20 25 30 Cis-splicing 50 ag Cis-splicing 2 20 25 30 25 ag 20 25 30 25 ag Cis-spliced (303 bp)

(bp) 500 200 ← Trans-spliced 200 (799 bn) 1 10 11 12 M 13 14 15 Σ

Cis-spliced (303 bp)

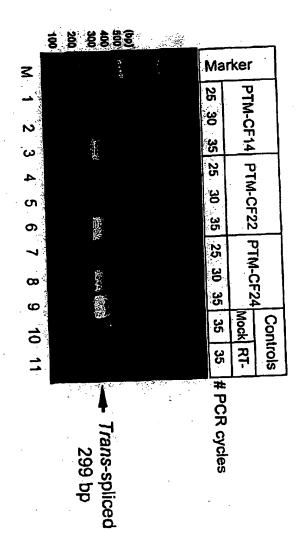
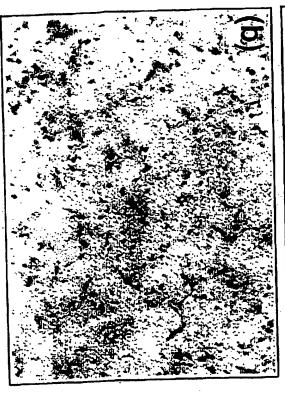


Figure 38B

2129

PAGE . 20



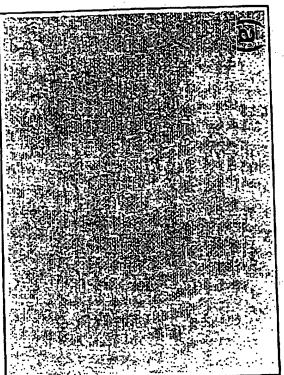
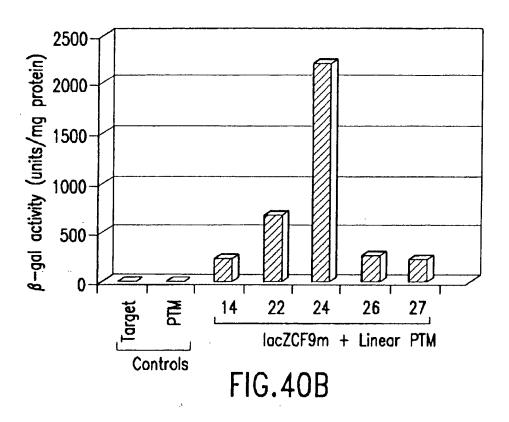
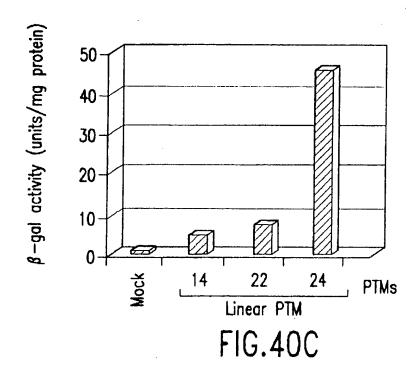


Figure 40A





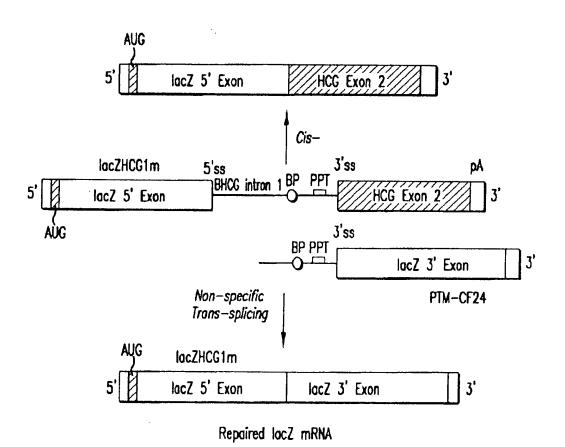
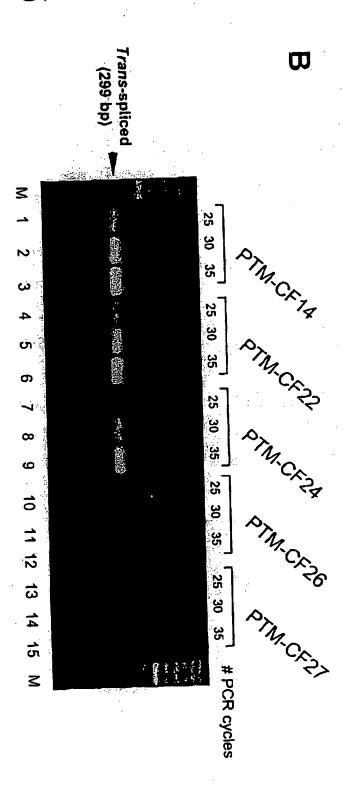


FIG.41A





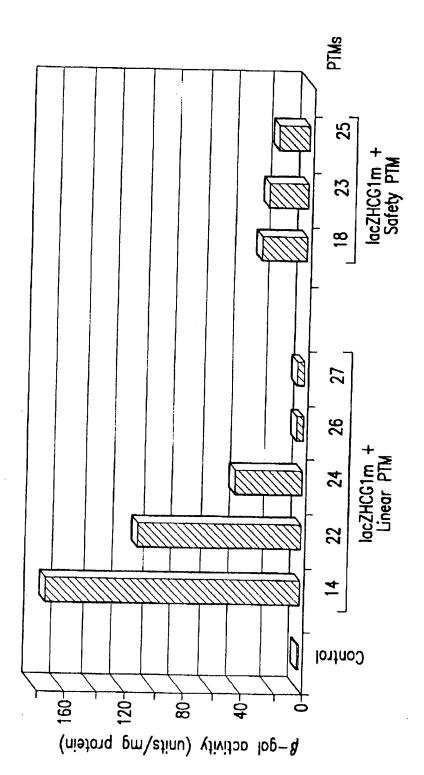


FIG. 41C

153 bp PTM24 Binding Domain:

Nhe I

CCTAGC—AATAATGACCAAGCCGCCCCTCAGGATTCACTTGCCTCCAATTATCATCCTAAGCAGAAGTGTATA

TTCTTATTGTAAAGATTCTATTAACTCATTTGATTCAAAATATTTAAAATACTTCCTGTTTCACCTACTCTGCTATGC

Sac II

FIG.43A

13.00 6

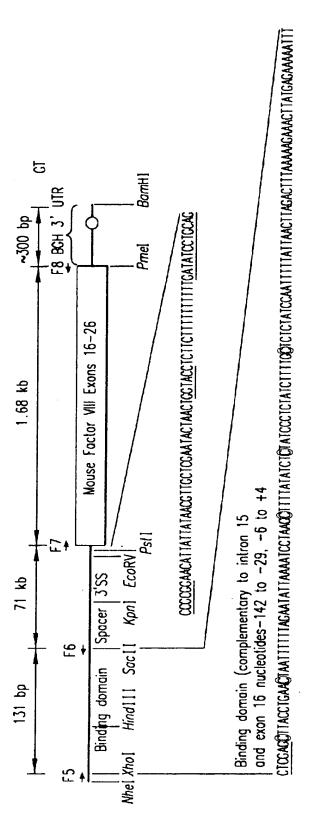


FIG.44A

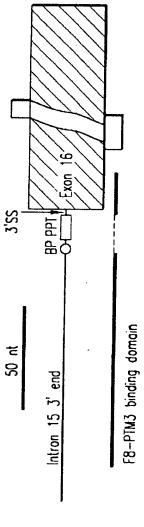


FIG.44B

Italicized=Spacer+PPT+BP+AG dinucleotide

7035212035

**CTTTAAAAAGAAACTTATGAGAAAA**TTT*ČČČČČSAACATTATTATAACGTTGCTG*CAA*TACTAACTGGTAC* TATTAAAATCCTAAGCTTTTATATCTCTATCCCTCTATCTTTTGCTCTCTATCCAATTTTTATTAACTTAGA CTCTTCTTTTTTTGATATCCTGCAG

Nucleotide changes are shown in blue Boxed+Arrow=Iranscription Start Oval=Downstream elements Boxed=Cat box, TATA box Bold=Binding domain Chicken B-actin Promoter

CCCCCCTCCCCCCCCCCCCCCCCCTCTGACCCCCTTACTCCCCACAGGTGAG CGGCCCCACCCCCTTCTCCTCCCGCTGTAATTAGCCCTTCGTTTAATCACCGCT IGTTICTTTCTGTGGCTGCGTGAAGCCTTGACGGCCTCCCGGAGGAATTCGTA <u>Sequence not included in construct</u>

F13+F2=235+106=341 bp F13+F4=235+315=550 bp Exon 1 Intron 1(partial) 94 Extent of promoter in original construct Extent of promoter in above construct CBA promoter 77 CMV enhancer

Chicken Beta Actin Promoter (including exon 1 and part of intron 1)

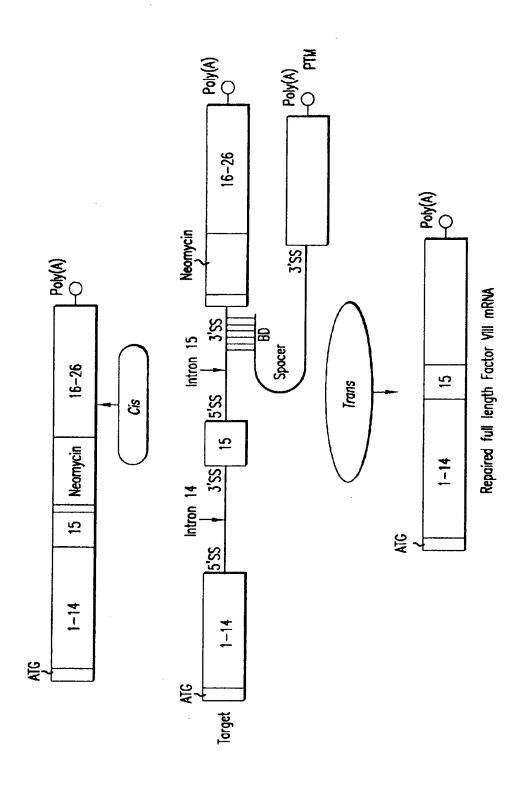


FIG. 44D

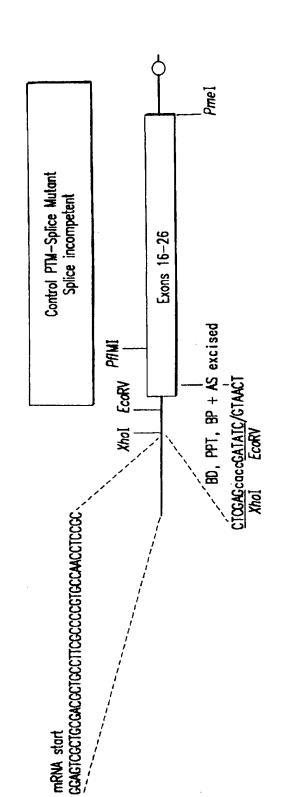


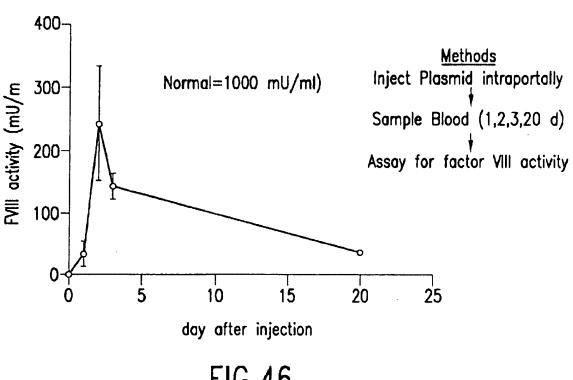
FIG 4

Excise TSD and part of exon 16 with XhoI and PfIMI and ligate in a PCR product that:
1) eliminates the TSD and splice acceptor site
2) inserts EcoRV adjacent to exon 16
3) restores the coding for exon 16

Method:

### Repair of Factor VIII Preliminary results from one experiment

FVIII activity in Exon 16 FVII—KO mice after IV PTM—FVII intraportal infusion  $(100 \mu gDNA)(n=3)$ 



**FIG.46** 

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Detailed structure of a mouse factor VIII PTM containing normal sequences for exons 16–26 and a C-terminal FLAG tag. BGH=bovine growth hormone 3' UTR; Binding domain= 125 bp.

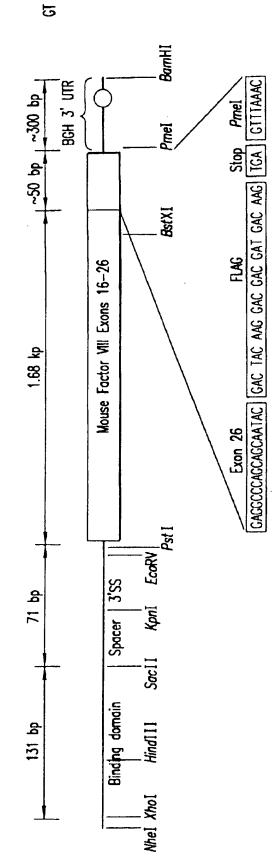
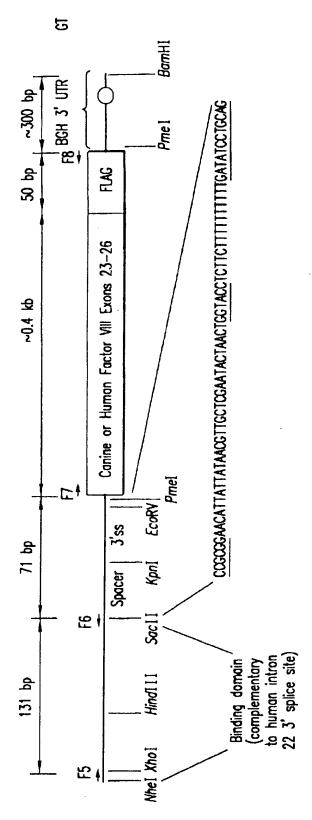


FIG. 47/



FLAG=C-terminal tag to be used to detect repaired factor VIII protein.